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BACE1 and Presenilin: Two Unusual Aspartyl Proteases Involved in Alzheimer's Disease

Diana-Ines Dominguez Dieter Hartmann Bart De Strooper

Neuronal Cell Biology and Gene Transfer Laboratory, Department of Human Genetics, KU Leuven and VIB4, Leuven, Belgium

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Abstract
Two enzymatic activities are required to generate the pathogenic β-amyloid (Aβ) peptide that accumulates in the brain of Alzheimer's disease patients. Both activities are carried out by two unusual aspartyl proteases known as β- and γ-secretase. Their therapeutic inhibition appears, therefore, a promising strategy to treat the disease. Transgenic mouse models in which the genes encoding the secretases have been ablated offer an invaluable tool, on the one hand, to gain more insights into the biological function of these proteases and, on the other hand, to predict the consequences that might be associated with enzyme inhibition in vivo.

Two unusual aspartyl proteases are implicated in Alzheimer's disease (AD). These are BACE1 [1] and the presenilins (PS) 1 and 2 [2] and they perform the two consecutive cleavages of the amyloid precursor protein (APP) required to generate the toxic β-amyloid (Aβ) peptide. First, BACE1 cuts APP, a type I transmembrane protein, at the β-site in the ectodomain 28 amino acids from the transmembrane region. BACE1 is an aspartyl protease that is unusual in that it contains a carboxy-terminal extension that harbors a transmembrane domain [3–6]. The enzyme is by itself necessary and sufficient to perform the cleavage. The BACE1-generated APP C-terminal fragment that remains membrane-bound is subsequently cleaved by a PS-containing γ-secretase complex at the γ-site within the transmembrane domain [7]. Although several membrane proteins have been shown to be processed within their transmembrane regions, it is not yet clear how hydrolysis can take place within the hydrophobic milieu of the membrane [8–11]. In addition to this unusual enzymatic property, PS-dependent cleavage, contrary to β-cleavage, requires the function of at least three additional proteins, Nicastrin [12], Pen-2 [13] and Aph-1 [14], that together with PS form the active γ-complex [15–17]. Despite the fact that direct proof that purified PS functions as a protease in vitro is still missing, many lines of evidence point strongly to PS as genuine aspartyl proteases [discussed extensively in 16, 18, 19]. The role played by the other components of the γ-complex in the cleaving reaction remains to be determined.

Alternatively to the BACE1/PS or β/γ-processing of APP that is known as 'amyloidogenic' since it results in Aβ formation, APP can be cleaved by α/γ-secretases in a nonamyloidogenic pathway. α-Secretases are membrane-bound metalloproteases that belong to the ADAM (a disintegrin and metalloprotease) family [20–22]. They cleave

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Diana-Ines Dominguez
Molecular Genetics Section, VIB4, Departement Menselijke Erfelijkheid, O. & N.
Herestraat 49, bus 602, BE–3000 Leuven (Belgium)
Tel. +32 16 345878, Fax +32 16 347181
E-Mail Diana-Ines.Dominguez@med.kuleuven.ac.be

APP between the amino acids lysine 16 and leucine 17 of the Aβ domain (numbers are given considering the first amino acid of Aβ as position 1), therefore limiting Aβ formation. The membrane-bound carboxy-terminal fragment of APP is subsequently processed within the transmembrane domain by γ-secretase resulting in the secretion of p3, a small peptide that lacks the amino-terminal 16 amino acids of Aβ.

γ-Secretase as Therapeutic Target

Because the activity of β- and γ-secretases is required to generate the pathogenic Aβ peptide, inhibitors of these enzymes are potential therapeutic drugs to treat AD. Therefore, understanding the biological functions of these two unusual proteases and defining their natural substrates is enormously interesting not only from a purely basic scientific point of view, but in addition it is also crucial for the development of AD therapies aimed at lowering Aβ.

The physiological role of PSs has been extensively investigated in *Caenorhabditis elegans*, *Drosophila melanogaster* and *Mus musculus*. Deficiencies in PS genes cause severe lethal phenotypes that closely resemble those observed upon inactivation of the Notch signaling pathway [23]. Even partially deficient PS1 ± PS2–/– mice that display an overall reduction in γ-secretase activity develop in adulthood a strong autoimmune phenotype [24]. The similarity in phenotypes of PS- and Notch-deficient animals can be explained by the fact that Notch receptors are substrates of γ-secretase and γ-cleavage is required to release the Notch intracellular domain from the membrane, which subsequently translocates into the nucleus and regulates gene transcription. The Notch pathway is responsible for complex cell fate decisions that occur during embryogenesis but also in adult life during T cell differentiation [25, 26] and neurite remodeling in the central nervous system [27]. Moreover, Notch and APP are not the only substrates for γ-secretase; in fact there is a growing list of type I transmembrane proteins that are processed by PS within their transmembrane regions. All the above data suggest that therapeutic inhibition of γ-secretase may lead to undesirable toxic side effects and, indeed, administration of a potent γ-secretase inhibitor to mice, beyond the expected decrease in plasma and brain Aβ levels, also resulted in marked defects in lymphocyte development and in the intestine villi and mucosa, most probably due to an inhibition of Notch processing [28]. The possibility, however, still exists of developing alternative drugs that rather than targeting the catalytic site of γ-secretase and thus affecting the cleavage of all substrates would specifically act on APP cleavage [81].

β-Secretase as Therapeutic Target

Unlike PS, genetic ablation of the BACE1 gene in mice is not associated with any gross phenotype [29–31], even in aged animals [32]. The only phenotype described thus far is some indicator of anxiety detected recently in more specific behavioral tests [33]. Moreover, BACE1 deficiency could prevent the learning and memory impairments and the cholinergic dysfunction observed in a transgenic mouse model for AD [34]. Whereas all these data highlight the therapeutic potential of BACE1 inhibition, important questions still need to be addressed to better predict the functional consequences (if any) of this action.

Several lines of evidence suggest that APP is not the only and probably not the main substrate for BACE1. First, the two proteins localize to different subcellular compartments in polarized cells limiting the access of BACE1 to its substrate. Whereas most BACE1 goes apically, the bulk of APP is sorted basolaterally [35]. Artificially targeting APP to the apical surface was sufficient to increase β-processing and Aβ generation. Similarly, the efficiency of BACE1 cleavage could be improved in nonpolarized cells by increasing the relative exposure of the substrate APP to BACE1 via its targeting to the endosomal compartment [36]. Second, the cleavage site in APP is not optimal and artificial sequences have been found that are processed far more efficiently not only than wild-type APP but also than APP carrying the Swedish familiar Alzheimer (FAD) mutation already known to be a better substrate for BACE1 [37]. It is, therefore, reasonable to assume that additional BACE1 substrates exist that are processed more efficiently than APP. Indeed two new substrates have been described for BACE1: P-selectin glycoprotein ligand-1 (PSGL-1) [38] and the sialyl transferase ST6Gal [39]. Both proteins are membrane-bound, as expected for a BACE1 substrate, and both have been ascribed a role in immune responses [40–42]. The evidence that they are substrates for BACE1 comes mainly from experiments in cultured cells, in conditions where both enzyme and substrate were overexpressed. Yet, whether these cleavages occur in vivo and their physiological significance, if any, remain to be determined. If PSGL-1 and ST6Gal are relevant physiological substrates of BACE1 and if their function is linked to the immune system, then the lack of any specific defect in BACE1-

deficient animals may simply reflect the specific pathogen-free conditions in which mice are housed. Alternatively, genetic redundancy and the activation of compensatory mechanisms could also account for the absence of phenotype in BACE1 null mice. No significant compensatory mechanism seems to be activated in brain for APP cleavage [29–31]; however, this result does not exclude the possibility that compensatory mechanisms operate to cleave other substrate(s). Resolving this issue is important because putative compensatory mechanisms activated during embryogenesis might not operate in the elderly when chronic BACE1 inhibition is considered as a treatment for AD.

Shortly after the discovery of BACE1, a homologous gene has been identified by database search and named BACE2 [6, 43–45]. BACE2 encodes a membrane-bound aspartyl protease that is 68% similar to BACE1 at the amino acid level and that can process APP at the β-site. BACE2 is then the nearest BACE1 homologue, but besides their amino acid homology and their common structural organization, the two enzymes differ in their tissue distribution [45–47], subcellular localization [48] and substrate specificity [45, 48, 49]. BACE2 is ubiquitously expressed in fetal and adult tissues, although the enzyme levels in brain are low [44, 45, 47]. Whether BACE2 affects, positively or negatively, the Aβ pool is a subject of debate. A positive contribution of BACE2 to brain Aβ levels has been suggested in two pathological conditions. First, because the BACE2 gene is located in the Down syndrome critical region of chromosome 21, it has been speculated that upregulation of BACE2 expression could be at least partially responsible for the higher levels of Aβ and the development of the AD-like neuropathology associated with this syndrome [43, 47, 50]. Second, based on the observation that BACE2 cleavage of APP at the β-site is more efficient when APP carries the Flemish FAD mutation, it has been proposed that BACE2-mediated APP cleavage might play a role in the development of AD in individuals carrying this mutation [45]. A number of observations suggest, in contrast, that BACE2 would function in vivo as an antiamyloid protein that would limit the amount of Aβ generated by BACE1. Such a property would be explained by the capacity of BACE2 to cleave APP within the Aβ region. In fact both BACE1 and BACE2 cleave APP at internal sites within the Aβ domain. BACE1 cleaves between tyrosine 10 and glutamic acid 11 (β11 position) and as a consequence of this cleavage an N-terminally truncated Aβ species is generated that is more prone to aggregation than full-length Aβ [51]. The internal BACE2 cleavage site is between residues phenylalanine 19 and 20 and BACE2 cleavage at this position is more efficient than at the β1-site [45, 48, 49]. If BACE2 cleaves preferably at this position in vivo, then BACE2 coexpression with BACE1 would result in decreased Aβ secretion. Indeed it has been shown that BACE2 overexpression reduces Aβ levels [44–46, 48] whereas BACE2 downregulation by RNAi elevates Aβ secretion [52]. These observations lead to the suggestion that BACE2 does not function in vivo as a β-secretase but rather as an α-like secretase that precludes Aβ formation [48, 49, 52, 53].

Despite some differences in substrate specificity, BACE1 and BACE2 cleave similar sequences and respond similarly to mutations introduced at the β-site in APP [45]. It is, therefore, likely that substrate-based inhibitors for BACE1 will also affect BACE2 function. This has important implications from a therapeutic point of view. On the one hand, if BACE2 negatively regulates Aβ levels, its nonselective inhibition could counteract the effect of BACE1 inhibition. On the other hand, although the natural substrate(s) for BACE2 are not known, the fact that the mRNA is widely expressed in fetal and adult tissues suggests it might fulfill essential functions. Moreover, whereas long-term BACE1 disruption does not seem to be associated with any gross phenotypic alteration, the physiological consequences of blocking both BACEs are thus far not known. BACE1 and BACE2 might process common substrates in vivo that have not yet been identified and this overlapping function(s) might be unique to these enzymes. The generation of BACE2- and BACE1/BACE2-deficient mice will help to address these questions.

Alternative Approaches for BACE1 Modulation

Alternatively to the nonselective inhibition of BACE1 activity, strategies can be envisioned that may target other aspects of BACE1 metabolism. Considerable progress has been made in our understanding of BACE1 biology since its initial discovery at the end of 1999 [3–6, 54]. In particular, some modes of modulating BACE1 protein levels as well as enzymatic activity have been proposed that might operate in vivo.

BACE1 protein levels and activity are increased in brains of patients with AD [55–59]; however, the levels of BACE1 transcript seem to be comparable in AD and nondemented controls [56, 60–62]. This suggests that BACE1 expression is regulated posttranscriptionally through a mechanism that is altered in AD. In this respect, the BACE1 transcript contains a 5′-untranslated region (5′-UTR) quite unusual for a cellular mRNA. It is more than 400 nucleotides long, contains three short open reading

frames and has a high GC content and hence the potential to fold into a stable secondary structure [63]. A ribosome-shunting mechanism has been proposed to explain how the translational machinery can access the BACE1 start codon. Ribosome shunting involves the recruitment of ribosomes in a cap-dependent manner and their subsequent nonlinear migration in which part of the 5′-UTR is skipped and ribosomes are directly translocated to a site at or close to the start codon of the major open reading frame [64]. The efficiency of shunting seems to be cell type-dependent, suggesting that cell-specific factors are involved in the modulation of BACE1 mRNA translation and raising the possibility that the process is altered in AD [63]. Identifying the mechanism of BACE1 mRNA translation and the factor(s) involved might reveal novel therapeutic targets to control BACE1 expression.

The increase in BACE1 protein observed in brains of AD patients can alternatively be explained by a decrease in protein turnover. There is thus far no solid indication that BACE1 protein is stabilized in AD brains, but a hypothesis can be drawn based on some data from cell culture studies. BACE1 is a rather stable protein with a half-life of about 16 h [65, 66]. Higher levels of the lipid second messenger ceramide in cultured cells led to stabilization of BACE1, which in turn resulted in an increase in BACE1-dependent APP products [66]. Because ceramide controls several biochemical events and its levels are elevated in AD brains, the authors propose that ceramide regulates Aβ generation in vivo by affecting the steady-state levels of BACE1 protein. Although more data are required to support this conclusion, the possibility that a signal transduction pathway having ceramide as second messenger can control BACE1 stability is quite appealing. Clearly further research is needed to identify the components of such a pathway and their modes of regulation.

In addition to the modulation of the levels of BACE1 protein, factors that directly affect enzyme activity have also been identified. Several lines of evidence suggest that β-secretase cleavage of APP takes place in rafts, specialized membrane microdomains rich in cholesterol and sphingolipids. First, cholesterol depletion that results in raft disruption caused a drastic decrease of β-cleavage in hippocampal neurons [67] and a strong increase of α-cleavage in various peripheral and neural cell lines [68]. Conversely, exposure of neurons and glial cells to cholesterol decreased α-secretase-mediated cleavage of APP [69]. Second, BACE1 protein has been shown to localize partially into rafts [70]. Finally, increasing the association of BACE1 with lipid rafts by either artificially adding a GPI anchor at the C-terminus of BACE1 ectodomain [71] or by antibody cross-linking [72] was sufficient to stimulate β-processing. Therefore, affecting the association of BACE1 with rafts appears as an alternative to modulate the efficiency of β-cleavage. This could be theoretically achieved in an indirect way by modulating cholesterol levels in the brain, and indeed cholesterol-lowering drugs are already being tested in clinical trials. The rational for using such drugs came originally from several epidemiological studies that showed a correlation between high blood cholesterol levels and a higher risk of developing AD [73–75]. Moreover, patients that have been treated with cholesterol-lowering statins were somehow protected against the disease [76, 77]. The protective role of statins in AD, although not definitively demonstrated, could then be the consequence of their indirect effect on BACE1 activity. An alternative and more direct way of modulating BACE1 compartmentalization into lipid rafts could theoretically be achieved via modulation of its interaction with proteins responsible for the localization of BACE1 into these microdomains. Although very speculative at this stage, at least two proteins have been proposed to play such a role: phospholipid scramblase 1 [78] and a GPI-anchored protein [79].

A novel and promising approach to control BACE1 activity has recently been described. BACE1 has been shown to bind to heparan sulfates (HS) both in vivo and in vitro and this binding resulted in inhibition of enzyme activity [80]. The inhibitory properties of the HS depended on the saccharide size and specific structural characteristics. HS inhibition in cultured cells was specific for β-cleavage of APP since no effect on α-cleavage was detected. The authors could show that HS function by blocking the access of the substrate to the enzyme's active site. Interestingly, inhibition of cellular HS synthesis resulted in a concomitant increase in BACE1 activity. These data are preliminary and at present far from practical therapeutic applications, but the possibility of using a natural, direct inhibitor to control BACE1 function definitively deserves further investigation.

Perspectives

The activity of BACE1 and PS is required to generate the pathogenic Aβ peptide that accumulates in the brain of AD patients. Clearly, blocking their activities appears to be a promising therapy to treat the disease. Transgenic mouse models in which the genes encoding the secretases have been ablated offer an invaluable tool, on the one hand, to gain more insights into the biological function of

these proteases and, on the other hand, to predict the consequences that might be associated with enzyme inhibition in vivo.

Genetic ablation of the PS genes in mice helped to identify the function of these enzymes in the Notch signalling pathway as well as their absolute requirement for the cleavage of Notch and APP within their transmembrane regions. The biological roles of Nicastrin, Pen-2 and Aph-1 as components of γ-secretase or their possible extra γ-secretase function remain to be determined. Several paralogs and alternatively spliced variants of at least PS and Aph-1 have been identified suggesting that γ-secretase is not an homogenous activity and the availability of mice deficient in γ-secretase components will help address these issues. Nicastrin- and Pen-2-deficient mice have been generated and they exhibit a phenotype consistent with inhibition of the Notch pathway. We are now awaiting the data for Aph-1-deficient mice.

The absence of any gross phenotype in BACE1 null mice converts this enzyme into a promising drug target. Pharmacologic inhibition of BACE1 does not appear, however, as straightforward as predicted. Moreover, active site inhibitors are likely to affect in addition BACE2 function. The generation of BACE2 and BACE1/BACE2 double knockout mice is, therefore, crucial to help us predict the consequences of blocking BACE function in vivo and to gain more insights into the biological functions of these two proteases.

Other alternatives to the active site inhibitors for PS and BACE1 can be envisioned that target other aspects of the enzyme metabolism, including for example their interaction with the substrate, their localization in specific subcellular sites where cleavage takes place or the modulation of the expression of the enzymes. Preliminary data that such approaches are feasible are available and energy should be dedicated to their further investigation.

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Correspondence and requests for materials should be addressed to R.Y. (e-mail: riqiang.yan@am.pnu.com) or M.E.G. (e-mail: mark.e.gurney@am.pnu.com).
Sequences are deposited in GenBank under the following accession numbers: human Asp1, AF200342; human Asp2, AF200343; mouse Asp2, AF200346; human Asp3, AF200343; and human Asp4, AF200345.

Purification and cloning of amyloid precursor protein β-secretase from human brain

Sukanto Sinha, John P. Anderson, Robin Barbour, Guriqbal S. Basi, Russell Caccavello, David Davis, Minhtam Doan, Harry F. Dovey, Normand Frigon, Jin Hong, Kirsten Jacobson-Croak, Nancy Jewett, Pamela Keim, Jeroen Knops, Ivan Lieberburg, Michael Power, Hua Tan, Gwen Tatsuno, Jay Tung, Dale Schenk, Peter Seubert, Susanna M. Suomensaari, Shuwen Wang, Donald Walker, Jun Zhao, Lisa McConlogue & Varghese John

Elan Pharmaceuticals, 800 Gateway Boulevard, South San Francisco, California 94080, USA

Proteolytic processing of the amyloid precursor protein (APP) generates amyloid β (Aβ) peptide, which is thought to be causal for the pathology and subsequent cognitive decline in Alzheimer's disease. Cleavage by β-secretase at the amino terminus of the Aβ peptide sequence, between residues 671 and 672 of APP, leads to the generation and extracellular release of β-cleaved soluble APP[1], and a corresponding cell-associated carboxy-terminal fragment. Cleavage of the *C*-terminal fragment by γ-secretase(s) leads to the formation of Aβ. The pathogenic mutation K670M671 → N670L671 at the β-secretase cleavage site in APP[2], which was discovered in a Swedish family with familial Alzheimer's disease, leads to increased β-secretase cleavage of the mutant substrate[3]. Here we describe a membrane-bound enzyme activity that cleaves full-length APP at the β-secretase cleavage site, and find it to be the predominant β-cleavage activity in human brain. We have purified this enzyme activity to homogeneity from human brain using a new substrate analogue inhibitor of the enzyme activity, and show that the purified enzyme has all the properties predicted for β-secretase. Cloning and expression of the enzyme reveals that human brain β-secretase is a new membrane-bound aspartic proteinase.

β-cleaved soluble APP (β-sAPP) was detected in membranes isolated from 293 cells stably overexpressing the 'Swedish' mutation, SweAPP751, by western blot analyses using the β-cleaved soluble APP (β-sAPP)-specific antibody Sw192 (ref. 4). Incubation of the membranes at pH 5.5 led to an increase in the cell-associated β-sAPP, and the appearance of a faster migrating species (Fig. 1). Treatment with O-glycanase resulted in the co-migration of both immunoreactive bands at the size of the lower band, which indicated that the smaller species resulted from β-cleavage of membrane-associated *N*-glycosylated immature APP (data not shown). These results are consistent with the specific cleavage of the full-length APP at the β-cleavage site by a membrane-bound proteinase activity. The membrane-bound β-cleavage activity exhibited a preference for acidic pH, with an optimum value of pH 5.5. Co-incubation with class-specific protease inhibitors, such as pepstatin, E-64 or phenylmethylsulphonyl fluoride, did not affect the generation of the β-cleaved APP (data not shown). Washing the membranes with 0.1% saponin under hypotonic conditions did not lead to loss of the membrane-associated β-cleavage enzyme activity (Fig. 1); therefore, we extracted P2 membranes[5] in 0.1% Triton X-100, 0.1% Brij-35 or 0.1% β-octylglucoside to test the solubility of enzyme activity. The soluble supernatant fractions were assayed for β-cleavage activity, on an exogenous recombinant substrate, MBPC125Swe. Specific β-cleavage was detected only in the Triton X-100 extracts.

We analysed various tissues and cell lines for β-cleavage activity, by extracting P2 membranes from each source with 0.2% Triton X-100 and assaying for β-cleavage (Fig. 2a). Human and mouse brain, and brain regions had uniformly high levels of enzyme activity, whereas little activity was detected in other tissues. In different cell lines, neurons had the highest level of enzyme activity, whereas 293, Cos and Chinese hamster ovary (CHO) cells had lower levels. Cells

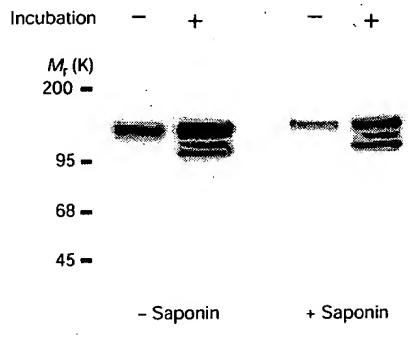

Figure 1 Endogenous substrate cleavage by β-secretase in P2 membranes. Membranes were prepared from 293 cells stably transfected with APP751, and either extracted with 0.1% saponin (+ saponin) or used directly (– saponin). Membranes in 0.1 M sodium acetate, pH 5.5 and 2% DMSO were either incubated (+) or solubilized without incubation (–). Samples were analysed by immunoblotting with the β-cleavage-specific 192sw antibody. M_r, relative molecular mass.

tionation approach. Sinha and colleagues[5] established that the β-secretase is a membrane-associated aspartyl protease with an optimal pH of 5.5. They then observed, like the other groups[2,4], that this presumably aspartyl-like activity is unusual in that it is not inhibited by pepstatin, a typical aspartyl-protease inhibitor. But to purify BACE, Sinha and colleagues needed a molecular hook. They designed several variants of the APP sequence, spanning the β-site, including so-called transition-state analogues. Such analogues 'freeze' a bound protease in the act of cleaving the substrate. Sinha *et al.* then tested their analogues against crude preparations of human brain containing abundant BACE activity. They found that one amino-acid substitution, from aspartate to valine with a statine analogue, at position +1 (that is, on the carboxy-terminal side of the cleavage site) resulted in a potent inhibitor with a half-maximum inhibition at 30 nM. Using this molecular hook, Sinha and colleagues pulled out their candidate protease from human brain extracts, with a 300,000-fold enrichment.

Finally Hussain and colleagues[3], like Yan *et al.*, named their β-secretase Asp2 — suggesting they have more than one candidate protease. However, Hussain *et al.* did not reveal in their paper how they obtained their cDNA clone from their proprietary expressed sequence tag (EST) database. They did show, however, that point mutations in Asp2 (or BACE) at both of its two active sites (the aspartic acid–serine/threonine–glycine sequence) mean that it can no longer process APP to Aβ.

Ever since it became clear that proteases chop APP down to Aβ, these proteases have been prime targets for drug discovery. In the absence of molecular targets, cellular reporter systems have been used to develop compounds that reduce the amount of Aβ produced, and we may soon see the first clinical trials of these drugs. But the isolation of BACE means we can now screen for drugs that act directly on the target protease. Future structural information from X-ray diffraction studies of BACE with a bound inhibitor might give valuable insights into the design of new structural classes of inhibitors.

Several challenges remain, however. The BACE and its homologue BACE-2 belong to a new class of membrane-bound aspartyl proteases. Are there other BACE homologues? And, if so, will these have to be considered for selectivity screens in drug-optimization studies? We also do not know which other precursor molecules or cellular processes depend on proper BACE activity. Transgenic mice with these genes knocked out, either conditionally or totally, will be very useful for resolving such questions. Another problem is the subcellular location of BACE, in the lumen of the Golgi body and endosomes (Fig. 1). This means that inhibitors will have to cross at least two lipid bilayers — a formidable penetration hurdle for even small-molecular-weight compounds. Moreover, any BACE inhibitor has to pass the blood–brain barrier to find its target in neurons. New compounds will therefore need to have excellent pharmacokinetic properties.

Despite all of this, the identification of the β-secretase means that the path towards specific inhibitors is now set, and it is time not only to test the amyloid hypothesis ('*in vivo veritas*'), but to find a way of halting this dreadful disease. ■

Bart De Strooper is at the Flanders Interuniversitary Institute for Biotechnology and K. U. Leuven, B-3000, Leuven, Belgium.
e-mail: Bart.Destrooper@med.kuleuven.ac.be
Gerhard König is at Bayer AG, Pharma Research CNS, 42096 Wuppertal, Germany.
e-mail: gerhard.koenig.gk@bayer-ag.com

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Chemical physics

Ultrafast relaxation in water

Abraham Nitzan

When a molecule is excited, where and how fast does its excess energy go? The answer to this question is a prerequisite for understanding and predicting the course of many chemical and biological processes. Chemical reactions can take place only when enough internal energy has accumulated in the molecule. The study of chemical reactions is therefore intimately connected with the study of energy-relaxation processes that compete with the chemical reaction channel. Intermolecular excitation transfer is one such process that has been studied for more than half a century. A paper by Woutersen and Bakker on page 507 of this issue[1] may force us to re-examine some of our notions about this important relaxation pathway.

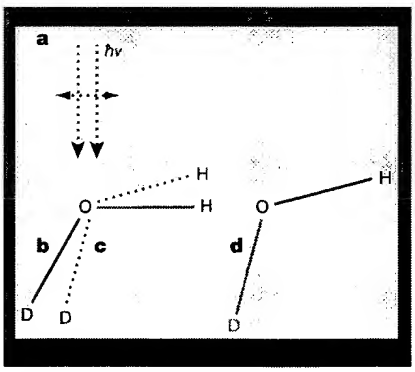


Figure 1 **Intermolecular energy transfer. Woutersen and Bakker[1] measure energy transfer in pure H_2O and in mixtures of HDO dissolved in D_2O using two infrared pulses. A pump pulse, polarized as shown in a, excites the OH bond of the HDO molecule, b. The induced rotational anisotropy can relax either by rotation of this molecule to configuration c, or by energy transfer to another molecule, d. Averaging over all final orientations amounts to loss of anisotropy, which is monitored by the probe pulse (not shown).**

When an isolated atom is optically excited it can relax to the ground state only by emitting radiation. In large molecular systems many degrees of freedom compete for the excitation energy and the winner rarely takes all. After a molecule is excited in solution, much of its energy usually ends up as increased solvent thermal motion, and can be regarded as wasted. Chemical interest often lies in other relaxation channels, for example electron transfer or chemical bond breaking. But even pathways that eventually lead to wasted thermal energy, such as intermolecular excitation transfer, can be of great interest at intermediate timescales. Knowing how energy flows between different molecular modes *en route* to complete relaxation can suggest ways to direct it to more useful channels, similar to harnessing the water flow in a river for useful work.

Intermolecular excitation energy transfer is the process by which one excited molecule, a donor, transfers its excess energy to another, an acceptor, leaving the latter in an excited state. This process continues until terminated by photon emission, chemical reaction or thermal relaxation. The intermolecular excitation pathway can be desirable or not, depending on your objective: it may obstruct an attempt to bring a molecule into higher excited states and it will destroy coherence that may otherwise help control a photochemical reaction. On the other hand it can provide ways to 'conduct' energy to where it is needed; an example is the use of sensitizers in photographic films in order to activate photoreactions in species that do not absorb natural light. Nature has also learned how to use these processes, for example in the light-harvesting complexes of photosynthetic systems[2].

The theory of such energy-transfer processes goes back to the well-known works of Förster[3] and Dexter[4]. The simplest Förster transfer mechanism is similar to the interaction between two electric dipoles. The rate of energy transfer, k, is described by $k = T^{-1}(r_o/r)^6$, where T is the lifetime of the excited state, r is the distance between the donor and acceptor, and r_o is a parameter called the Förster radius. This equation tells us that the rate of dipolar energy transfer behaves like r^{-6}. With increasing r, higher-order interactions (such as dipole–quadrupole, quadrupole–quadrupole and exchange interactions) decay much more rapidly than the dipole–dipole interaction, and are effective only at very small intermolecular distances.

How important is this mode of energy flow? It is significant only when its rate is comparable to or faster than other relaxation processes. The most important competing processes are intramolecular vibrational relaxation, where vibrationally excited molecules relax by transfer of energy within the molecule itself, and vibrational energy relaxation, where vibrational energy is transformed into solvent thermal energy. These processes are fast; relaxation of polyatomic molecules in condensed phases at room temperature occurs over a few picoseconds or less.

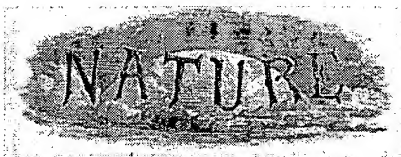
In contrast, vibrational energy transfer between molecules is generally believed to be too slow to be important. It is only expected to play a significant role for diatomic molecules or at cryogenic temperatures, where vibrational relaxation is relatively slow. Indeed, these are the conditions under which such processes have been observed in the past[5,6]. Contrary to such expectations, the experiment by Woutersen and Bakker[1] shows that resonant intermolecular energy transfer between OH bonds in liquid water is extremely fast. Moreover, it appears to be much faster than the vibrational energy relaxation of the OH group, which has recently been shown to have a short lifetime of 740 fs (femtoseconds)[7].

In their experiment, Woutersen and Bakker use two 200-fs infrared pulses: one relatively strong, linearly polarized 'pump' pulse to excite the OH groups and another, low-intensity pulse to probe this excitation. They use thin-layer samples of either pure water (liquid H_2O), or a mixture of HDO and D_2O (D is deuterium, a heavy isotope of hydrogen). The mixed samples make it possible to measure the dependence of the energy transfer rate on the OH concentration. Woutersen and Bakker measure the rotational anisotropy of the molecules as a function of the time delay between pump and probe. Rotational anisotropy is induced by the pump pulse, which excites molecules with specific orientations. This vibrational excitation can then relax either by rotational motion of the excited molecules or by energy transfer between molecules of different orientations (Fig. 1).

These two relaxation modes can be distinguished from each other for the low OH mixtures (that is, HDO dissolved in D_2O): energy transfer between HDO molecules depends on the concentration of this species, whereas their rotation does not. Therefore measuring the rotational anisotropy at different delay times as a function of HDO concentration yields the characteristic time for molecular rotation (four picoseconds), and more importantly the rate of intermolecular vibrational energy transfer between the OH groups. These results show that the Förster theory accounts well for the observed intermolecular vibrational energy transfer in HDO–D_2O mixtures and that the corresponding transfer rate is quite fast — in the range of a few picoseconds for molar concentrations of OH. With Woutersen and Bakker's technique, the transfer rate is measurable even though the competing processes of energy relaxation are very fast.

The real surprise comes from similar measurements in pure H_2O. Using the Förster results from mixtures of HDO in D_2O ($r_o = 2.1$ Å) to extrapolate to the intermolecular distance in pure water (2.8 Å) predicts an energy-transfer time in the range of a few hundred femtoseconds. But the observed intermolecular energy transfer in pure water takes place even faster than the experimental time resolution of ~100 fs. This is considerably faster than the 740-fs lifetime of the excited OH population, and makes intermolecular vibrational energy transfer one of the fastest relaxation processes ever recorded in water. This means that vibrational energy cannot be localized in water long enough to affect most chemical reactions. On the other hand it implies that water is an extremely good conductor of vibrational energy through its OH groups. It is even possible that this energy-transfer process could involve other molecules containing OH groups, so water may play an important role in protein dynamics when energy is transported between different molecules.

The failure of the dipolar Förster theory in H_2O is not unexpected because the OH groups are so close to each other that higher-order interactions come into play. But the observation that intermolecular vibrational energy transfer in water is so amazingly fast calls for a reassessment of vibrational energy transfer and relaxation in condensed phases. Many questions remain. For example, what is the actual rate of vibrational energy transfer in water? Is this behaviour peculiar to this liquid (perhaps it is associated with its special structural properties)? Previous studies from the same laboratory[7] have suggested that the fast vibrational energy relaxation of the OH bond in water is possibly associated



100 YEARS AGO

Two or three months ago reports were published in the daily press of the discovery of an electrical method of giving sight to the blind. It was alleged that Mr. Stiens had succeeded in constructing an electrical apparatus which performed all the functions of the eye and was an efficient substitute for it. Like many other newspaper reports of so-called scientific discoveries, this has proved to be without sound foundation. Mr. G. H. Robertson, who is himself afflicted with blindness, describes in the *Electrician* the results of personal inquiries into the matter with a member of the staff of our contemporary. In spite of several visits to Mr. Stiens, no experimental proof in substantiation of the claims which were put forward on his behalf was obtained, and the conclusion arrived at is that these claims are foundless. Life is so short and crowded with so many important duties that it is impossible to investigate the many sensational statements made by irresponsible interviewers, but we are grateful to any one who will take the trouble to examine some of the rumours which are put forward in the name of science.

From *Nature* 30 November 1899.

50 YEARS AGO

In British Astronomical Association Circular No. 312 some details are given regarding the two newly discovered satellites of Uranus and Neptune, respectively. Both were discovered by Gerard P. Kuiper during his search for new satellites with the 82-in. reflector of the McDonald Observatory, University of Texas. The new satellite of Uranus, now named Miranda, was discovered on February 16, 1948, magnitude 17, and is now known to have a period of about 33h. 56m. The motion is approximately circular and in the plane of the other four satellites. Neptune ii, for which the name Nereid has been proposed by the discoverer, was found on May 1, 1949, on plates exposed for forty minutes at the prime focus, with the mirror stopped down to sixty-six inches (*f*/5). Its magnitude was estimated to be 19.5, and later observations show that its period is about two years and that the plane of its orbit is within six degrees of the ecliptic. Kuiper says that, as Neptune could retain satellites nearly ten times as far away as Nereid, with periods up to about fifty years, further work is planned to cover the outer regions of the system.

From *Nature* 3 December 1949.

with its coupling to the nearest hydrogen bond. Is there a link between that and the present observations? If not, what is the origin of this extremely fast energy-transfer process? The need to answer these questions is our next challenge.

Abraham Nitzan is at the School of Chemistry, Sackler Faculty of Science, Tel Aviv University, Tel Aviv 69978, Israel.
e-mail: nitzan@post.tau.ac.il

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Neurobiology

Derailed axons get on track

Kai Zinn and Aloisia Schmid

How do growing axons in the central nervous system navigate through the dense jungle of cells and processes that they encounter on the way to their targets? On page 540 of this issue, Bonkowsky *et al.*[1] show that the choice of pathways for growth cones (the leading edges of growing axons) in the fruit fly *Drosophila melanogaster* is regulated by a receptor tyrosine kinase known as Derailed (Drl). These results further indicate that, rather than attracting growth cones to the right pathways, Drl causes them to be repelled from the wrong ones.

The array of axons in the embryonic *Drosophila* central nervous system has a ladder-like structure (Fig. 1). Anterior and posterior commissural tracts cross the midline in each body segment, and two longitudinal tracts extend the length of the embryo. Each of the roughly 300 neurons within a unit of this structure is thought to extend its axon along a genetically determined pathway. Most interneurons (neurons that synapse with other neurons) extend axons across the midline of the central nervous system, and attractive and repulsive factors that regulate this fundamental crossing decision have been identified[2,3]. But axon guidance at the midline involves more than just the decision whether or not to cross. Each commissure contains many distinct pathways, and the growth cone of a particular neuron always follows the same one. The unique sequence of navigational decisions made by its growth cone determines the complex and invariant shape of each axon in the central nervous system[4].

Although we are far from understanding how complete axon trajectories (such as those in Fig. 1) are determined, the results of Bonkowsky *et al.*[1] are an important step forward. These authors define how axons choose between the two major subdivisions of the crossing pathways — the anterior and posterior commissures. They show that Drl is normally expressed on axons that follow the anterior commissure, but not on those that take the posterior route[1,5]. Moreover, forced expression of Drl on specific axons that normally take the posterior commissure causes them to choose the anterior tract instead (Fig. 2).

Can Drl redirect any crossing axon into the anterior commissure? To address this question, Bonkowsky *et al.* simultaneously misexpressed the Commissureless (Comm) protein and Drl on a set of axons that normally never cross the midline (the thoracic Ap axons). Expression of Comm in neurons downregulates a repulsive signal from the midline that is transduced by the Roundabout (Robo) receptor. So, non-crossing axons that express Comm are diverted into pathways that cross the midline[6]. The authors found that when the thoracic Ap axons expressed only Comm, they crossed in the posterior commissure. But when they

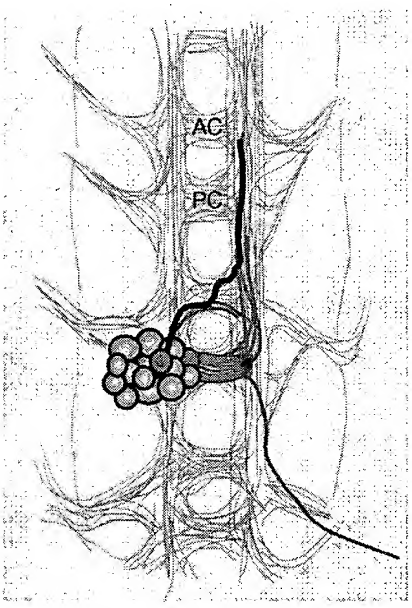

Figure 1 **Individual axons follow complex trajectories within the central nervous system (CNS). The axon scaffold of the fly CNS is drawn in grey. A specific neural lineage (from neuroblast 5–2) is indicated in brown; the lineage includes neurons that project in both the anterior commissure (AC) and the posterior commissure (PC). One AC neuron (red) — presumably a cell that expresses Drl — extends its axon (black) along the posterior edge of the AC. At the midline the axon veers anteriorly, grows along a bundle at the anterior edge of the AC, then turns into the longitudinal tract. (Modified from ref. 4.)**

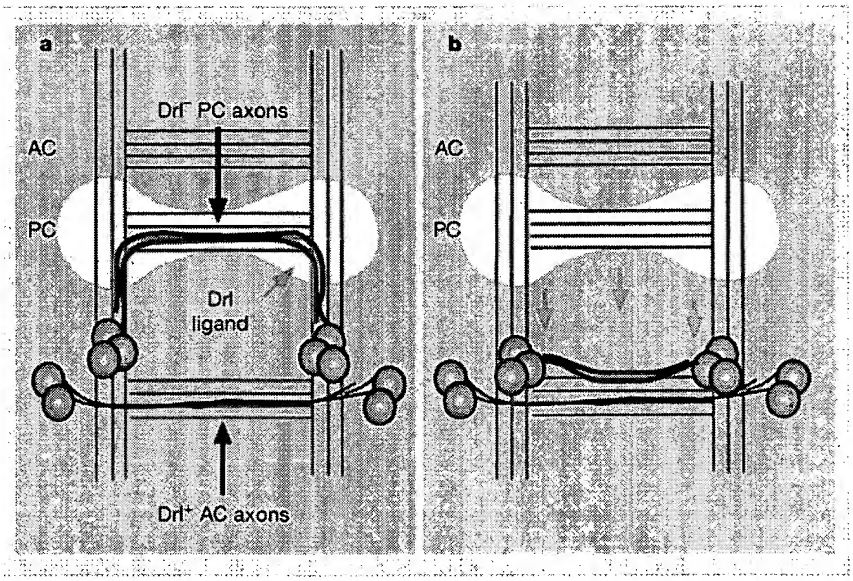

Figure 2 **Effects of Drl expression on axon guidance. Bonkowsky *et al.*[1] have found that expression of a protein called Drl controls pathfinding by causing growth cones to be repelled from the wrong pathway. a, Wild-type embryos. The AC (black) and PC (red) Eg axons cross the midline. The distribution of the putative Drl ligand is indicated by yellow shading, and the axon scaffold is in green. b, Forced expression of Drl in the PC Eg axons[1]. These axons are repelled by Drl ligand (grey arrows) and instead project across the AC.**

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Correspondence and requests for materials should be addressed to A.A. (e-mail: aertsen@biologie.uni-freiburg.de).

Membrane-anchored aspartyl protease with Alzheimer's disease β-secretase activity

Riqiang Yan*†, Michael J. Bienkowski†‡, Mary E. Shuck‡, Huiyi Miao*, Monica C. Tory‡, Adele M. Pauley§, John R. Brashler‖, Nancy C. Stratman§, W. Rodney Mathews¶, Allen E. Buhl‖, Donald B. Carter§, Alfredo G. Tomasselli#, Luis A. Parodi☆, Robert L. Heinrikson# & Mark E. Gurney§

** Cell & Molecular Biology, ‡ Genomics, # Protein Sciences, ‖ Pharmacology, ¶ Structural, Analytical & Medicinal Chemistry and § Neurobiology, Pharmacia & Upjohn, Inc., 301 Henrietta Street, Kalamazoo, MI 49007, USA*
☆ Bioinformatics, Pharmacia & Upjohn, Inc., Lindhagensgatan 133, S-11287 Stockholm, Sweden
† These authors contributed equally to this work

Mutations in the gene encoding the amyloid protein precursor (APP) cause autosomal dominant Alzheimer's disease[1–3]. Cleavage of APP by unidentified proteases, referred to as β- and γ-secretases[4–7], generates the amyloid β-peptide, the main component of the amyloid plaques found in Alzheimer's disease patients[8]. The disease-causing mutations flank the protease cleavage sites in APP and facilitate its cleavage. Here we identify a new membrane-bound aspartyl protease (Asp2) with β-secretase activity. The Asp2 gene is expressed widely in brain and other tissues. Decreasing the expression of Asp2 in cells reduces amyloid β-peptide production and blocks the accumulation of the carboxy-terminal APP fragment that is created by β-secretase cleavage. Solubilized Asp2 protein cleaves a synthetic APP peptide substrate at the β-secretase site, and the rate of cleavage is increased tenfold by a mutation associated with early-onset Alzheimer's disease in Sweden[3]. Thus, Asp2 is a new protein target for drugs that are designed to block the production of amyloid β-peptide peptide and the consequent formation of amyloid plaque in Alzheimer's disease.

Visual inspection suggests that the β- and γ-secretase cleavage sites in APP might be substrates for cleavage by aspartyl proteases, and indeed, cathepsin D cleaves synthetic β-secretase substrates[9]. This cleavage is facilitated by the KM → NL mutation, referred to as the 'Swedish' mutation, found in patients with early-onset Alzheimer's disease[10]; however, APP processing to amyloid β (Aβ) peptides occurs normally in hippocampal neurons cultured from cathepsin-D-null mice[11]. Nevertheless, it seemed plausible that the APP β- or γ-secretases could be as yet uncharacterized aspartyl proteases; therefore, we searched for new human enzymes of this mechanistic set. Sequencing of the *Caenorhabditis elegans* genome was nearing completion, which offered the possibility of enumerating the complete set of aspartyl proteases encoded in a simple metazoan genome, and using these as a bridge to human sequence databases.

Simple AWK scripts scanning for the D(S/T)G active-site motif, PROSITE and hidden Markov models were used to search the WormPep database of predicted *C. elegans* proteins. This revealed at least 10 candidate aspartyl proteases. Seven of these ten were found on a single chromosome, chromosome V (*F21F8.3*, *F21F8.4*, *F21F8.7*, *Y39B6B.G*, *Y39B6B.J*, *Y39B6B.H* and *T18H9.2*), and three each of these were found in the same cosmid clones (*F21F8* and *Y39B6B*), suggesting that they represent a recently evolved family of proteins that arose by ancestral gene duplication. Other homologous predicted genes were found in the same cluster (*F21F8.2*, *F21F8.6* and *Y39B6B.I*); however, these contain only a single DTG or DSG motif. Additional predicted aspartyl protease genes were found on chromosomes IV (*C11D2.2*) and X (*R12H7.2* and *H22K11.1*). Searches of vertebrate expressed sequence tag (EST) databases with the 10 *C. elegans* sequences identified 7 known and 4 new candidate aspartyl proteases. The new human sequences were numbered in order of their discovery (Asp1–4). *R12H7.2* and *H22K11.1* appear to be *C. elegans* homologues of cathepsin D. Most of the chromosome V aspartyl proteases had no clear vertebrate orthologues; however, one of these (*T18H9.2*) bridged to two unusual sequences (Asp1 and Asp2) which contained the less common DSG motif in the second active site. In turn, *C11D2.2* identified two additional sequences (Asp3 and Asp4) which have since been reported in the literature as napsins A and B[12].

The two predicted aspartyl protease sequences identified by *T18H9.2* were of greatest interest. Completion of their sequences by a combination of EST sequencing, 5′ rapid amplification of complementary DNA ends by the polymerase chain reaction, and library screening showed that both Asp1 and Asp2 had an unusual C-terminal extension containing a single predicted transmembrane domain (Fig. 1). Asp1 maps to human chromosome 21q22 within the Down's syndrome critical region, and Asp2 to chromosome 11q23–24. Northern hybridization to human tissue blots showed widespread expression of both Asp1 and Asp2. Both are expressed at the highest levels in pancreas. Asp2 is also expressed at high levels in brain, whereas Asp1 is expressed in brain at somewhat lower levels. *In situ* hybridization showed expression of Asp2 primarily in acinar cells of the exocrine pancreas, whereas faint hybridization was seen over neurons in hippocampus; however, we identified two Asp2 EST in a human astrocyte cDNA library indicating that Asp2 may be expressed in both neurons and glial cells. Transcripts for both Asp1 and Asp2 were expressed in human embryonic kidney 293 cells, human IMR-32 neuroblastoma cells and mouse Neuro-2a neuroblastoma cells, three commonly used cellular models of APP processing.

We used a panel of antisense oligomers to test the involvement of each of the four predicted aspartyl proteases in APP processing by a stable clone of HEK293 cells that had been engineered to process APP to Aβ peptides at high levels. These cells were transformed with a modified human APP695 cDNA containing the Swedish KM → NL mutation to which two lysine residues had been added to the C terminus (HEK/APP-Sw-KK cells). The KK motif greatly increases the processing and release of Aβ peptides but does not influence the ratio of Aβ42/(Aβ42 + Aβ40), nor alter the effect of

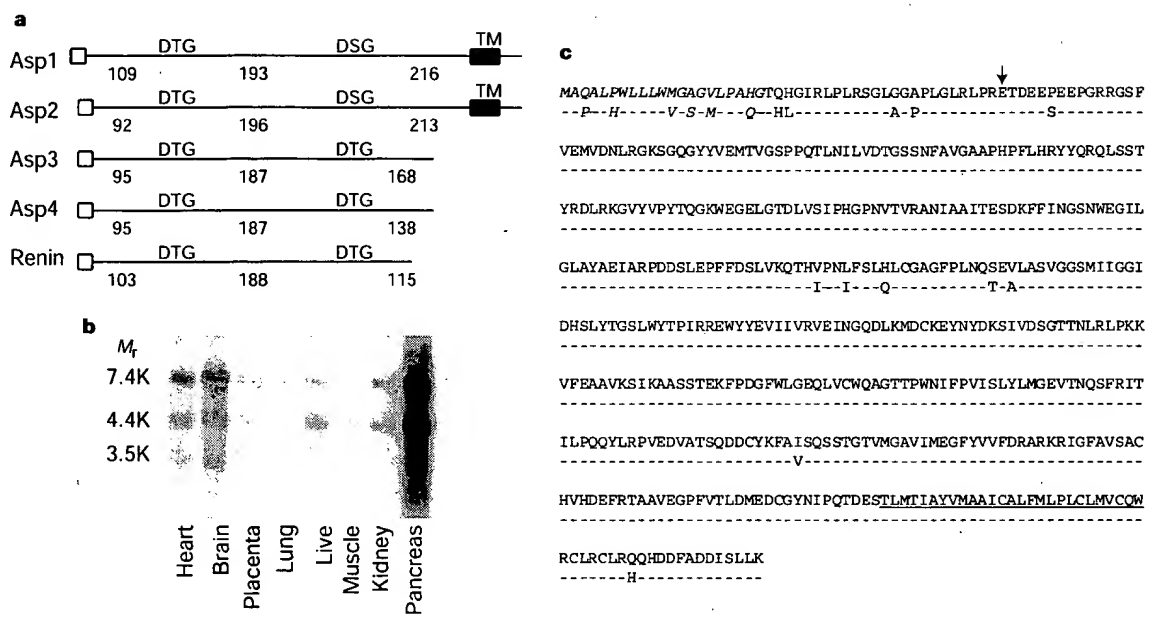

Figure 1 Asp2 functional domains, tissue distribution and amino-acid sequence. **a**, Alignment of the predicted primary structures of Asp1–Asp4 with human renin. Asp1 and Asp2 (51% amino-acid identity) have a DSG motif in the second predicted active site and possess an unusual C-terminal extension containing a single predicted transmembrane domain (TM). The predicted signal peptide for secretion (open box) and the number of residues in each domain is indicated. **b**, Tissue distribution of human Asp2 mRNA expression as shown by northern hybridization. The relative molecular mass (M_r) of each transcript is indicated. **c**, Clustal W sequence alignment of Asp2 from human (top line) and mouse (second line). The signal peptide is indicated in italics, the predicted transmembrane domain is underlined, and the active-site sequences are in bold. Arrow indicates the N terminus of purified recombinant Asp2 expressed in CHO cells.

the KM → NL or V717F mutations. These mutations increase total Aβ processing[4,5] or selectively increase the production of Aβ42[6], respectively. Thus, the HEK/APP-Sw-KK cells provide a sensitized background on which to screen for inhibition of Aβ processing.

Transfection of HEK/APP-Sw-KK cells with the panel of 16 antisense oligomers (four each targeting Asp1–Asp4) showed that only those oligomers targeting Asp2 considerably decreased the release of Aβ peptides into the medium. Two of the Asp2 antisense oligomers were chosen for resynthesis, as well as for synthesis of two additional oligomers containing the reverse sequence for use as controls. Their effects on transfected HEK/APP-Sw-KK cells are shown in Fig. 2. Both of the antisense oligomers targeting the human Asp2 transcript reduced Asp2 message levels, whereas the control reverse oligomers did not. Both also reduced the release of Aβ peptides into the culture medium. The inhibition of Aβ release ranged from 50% to 80% in many separate experiments and probably was dependent upon transfection efficiency. The antisense oligomers reduced the production of both Aβ40 and Aβ42 by roughly the same amount. The reduction of Aβ peptide production also was confirmed by immunoprecipitation and western blotting. This indicates that Asp2 may be involved directly or indirectly in the production of Aβ peptides and their release from HEK293 cells.

Because HEK293 cells derive from kidney, we extended the experiment to human IMR-32 neuroblastoma cells, which express all three APP isoforms[13] and which release Aβ peptides into conditioned medium at measurable concentrations[14], and obtained essentially identical results. The Asp2-1A and Asp2-2A antisense oligomers reduced Asp2 messenger RNA by 75% and 39%, respectively (quantitated using a TaqMan probe), whereas the reverse control oligomers had no effect. Correspondingly, release of Aβ40 and Aβ42 was reduced by 49 ± 2% and 42 ± 14% from cells treated with Asp2-1A, and by 43 ± 3 and 44 ± 18 with Asp2-2A ($P < 0.001$). Again, the reverse control oligomers had no effect.

Similar results were obtained in a murine system. Molecular cloning of the mouse Asp2 cDNA revealed a remarkable 98% amino-acid identity to human (Fig. 1c) and complete nucleotide identity at the sites targeted by the Asp2-1A and Asp2-2A antisense oligomers. In mouse Neuro-2A cells engineered to express APP-Sw-KK, the Asp2-1A antisense oligomer reduced the release of Aβ40 and Aβ42 by 70 ± 7% and 67 ± 2%, whereas a reduction of 61 ± 12% was seen for the release of both Aβ40 and Aβ42 from cells treated with Asp2-2A ($P < 0.001$). The reverse control oligomers had no effect. Thus, the three antisense experiments with HEK293, IMR-32 and Neuro-2a cells indicate that Asp2 is directly or indirectly involved in Aβ processing in both somatic and neural cell lines.

Treatment of HEK293/APP-Sw-KK cells with the Asp2 antisense oligomers had little effect on the release of total soluble APP (sAPP) from cells although they did appear to alter the ratio of sAPP isoforms released by either α- or β-secretase cleavage (Fig. 3). These cleavages generate species of soluble APP (sAPPα and sAPPβ, respectively), which contain different C termini which can be distinguished by the 6E10 monoclonal antibody that recognizes Aβ residues 1–16. As shown in Fig. 3b, no change in the release of total sAPP is shown on western blots developed with the 22C11 monoclonal antibody that reacts with an amino-terminal epitope of APP, whereas development with 6E10 shows an increase in sAPPα release from cells treated with either of the Asp2 antisense oligomers. An enzyme-linked immunosorbent assay (EIA) specific for sAPPα showed that release increased at least twofold, from 2.8 μg ml^{-1} to 6.7 μg ml^{-1} ($P < 0.005$), in cells transfected with the Asp2-2 antisense oligomer.

Cleavage of APP at either the β- or α-secretase sites also leaves C-terminal fragments containing the APP transmembrane domain and cytoplasmic tail. These contain 99 and 83 amino acids (CTF99 and CTF83), respectively (Fig. 3a). Indeed, in HEK293/APP-Sw-KK cells treated with Asp2 antisense oligomers, the amount of the CTF99 β-secretase product is reduced (Fig. 3c). Correspondingly, co-transfection of HEK293 cells or Neuro-2A cells with human Asp2 and APP-KK increases the production of CTF99 in comparison with cells co-transfected with APP-KK and empty vector DNA. Production of CTF99 is increased still further in cells co-transfected

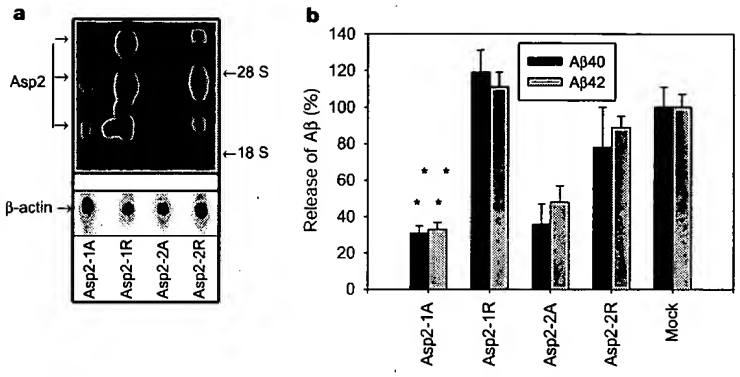

Figure 2 Asp2 antisense oligomers decrease amyloid β-peptide processing. **a**, Asp2 antisense oligomers (Asp2-1A and Asp2-2A) targeting two different sites on the Asp2 transcript specifically reduce Asp2 mRNA in transfected HEK/APP-Sw-KK cells as determined by northern hybridization, whereas control oligomers (Asp2-1R and Asp2-2R) with the reverse sequence lacked this effect. Arrows indicate the three Asp2 transcripts present in HEK293 cells. No change was seen in β-actin mRNA in transfected cells. **b**, Release of Aβ40 and Aβ42 from HEK/APP-Sw-KK cells, as measured by EIA, was reduced specifically by the Asp2 antisense oligomers (asterisks, $P < 0.001$). Antisense oligomers were transfected in quadruplicate cultures. Release was normalized against values for Mock-transfected cells treated with oligofectin-G only.

with constructs expressing Asp2 and APP-Sw-KK. Thus, Asp2 appears specifically to facilitate β-secretase cleavage of APP and this effect is enhanced by the Swedish KM → NL mutation. This is in contrast to the effects of presenilin-1 gene disruption, which specifically increases the accumulation of CTF99 in cultured mouse neurons because of inhibition of γ-secretase cleavage[15,16].

Effects of Asp2 on the production of Aβ peptides from endogenously expressed APP isoforms were assessed in HEK293 cells transfected with a construct expressing Asp2 or with the empty vector after selection of transformants with the antibiotic G418. Aβ40 production was increased in cells transformed with the Asp2 construct in comparison with those transformed with the empty vector DNA, the concentrations in conditioned medium were $424 \pm 45\,pg\,ml^{-1}$ and $113 \pm 58\,pg\,ml^{-1}$, respectively ($P < 0.001$). Aβ42 release was below the limit of detection by the EIA, whereas the release of sAPPα was unaffected, $112 \pm 8\,ng\,ml^{-1}$ versus $111 \pm 40\,ng\,ml^{-1}$. These results provide further support for the hypothesis that Asp2 functions in the processing and release of Aβ from endogenously expressed APP.

To determine the effects of Asp2 on the production of Aβ peptides from mutant APP, we transfected the two pools of cells with a panel of APP constructs. This showed that co-expression of Asp2 with APP or APP-VF increased Aβ40 release from cells by 44% and 36%, respectively ($P < 0.05$), and that this effect was magnified by addition of the KK motif (126% and 186%, respectively, $P < 0.001$), Fig. 4a. Consistent with other reports[6], the V717F mutation increased the production of Aβ42 relative to total Aβ peptides, which was further increased to 175% by co-expression of Asp2 ($P < 0.001$), but there was no change in the relative ratio, Fig. 4b, c. Thus, Asp2 had little effect on the choice of γ-secretase cleavage sites in transformed HEK293 cells.

Co-expression of Asp2 in HEK293 cells with constructs containing the Swedish KM → NL mutation did not have the same effect on Aβ production. Consistent with other reports[4,5], the Swedish mutation increased the production of both Aβ40 and Aβ42 with no change in their relative ratio (Fig. 4). However, when Asp2 was overexpressed in HEK293 cells, co-expression of APP-Sw caused a decrease in Aβ40 and Aβ42 release in comparison with control

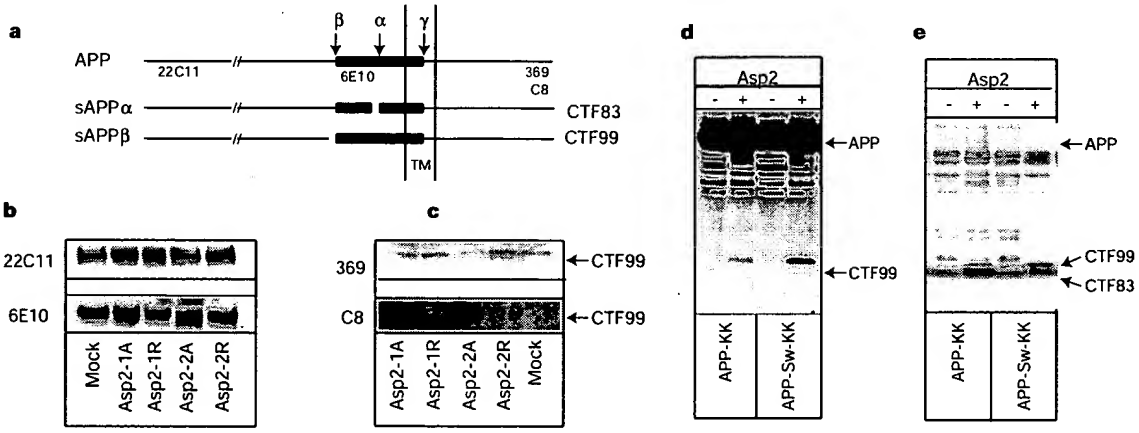

Figure 3 Asp2 increases production of the APP C-terminal β-secretase product. **a**, Illustration of the β-, α- and γ-secretase cleavage sites in APP and location of the 22C11, 6E10, 369 and C8 epitopes. The location of the Aβ peptide within APP is indicated (box). Processing at the α-secretase site cleaves the mid-region of the Aβ sequence and liberates the sAPPα ectodomain containing the 6E10 epitope, whereas the consequent 83-amino-acid C-terminal fragment (CTF83) retains the APP transmembrane domain (TM). Processing at the β-secretase site releases the sAPPβ ectodomain and creates a 99-amino-acid C-terminal fragment (CTF99) containing the 6E10 epitope. **b**, Equal amounts of conditioned culture supernatants from HEK/APP-Sw-KK cells were analysed on western blots developed with the 22C11 and 6E10 antibodies. Cultures were treated with the oligomers or transfection reagent as indicated. **c**, Equal amounts of protein from lysates of HEK/APP-Sw-KK cells were immunoprecipitated with either the 369 or C8 antibody as indicated, and analysed on western blots developed with 6E10 to identify CTF99. Cultures were treated with the oligomers or transfection reagent as indicated. **d**, Neuro-2A cells were co-transfected with either APP-KK or APP-Sw-KK, with or without Asp2 as indicated. Equal amounts of protein from cell lysates were analysed by western blot developed with 6E10 to detect APP and CTF99 (arrows). **e**, HEK293 were co-transfected with either APP-KK or APP-Sw-KK, with or without Asp2 as indicated. Equal amounts of protein from cell lysates were analysed by western blot developed with 369 antibody to detect APP, CTF99 and CTF83 (arrows).

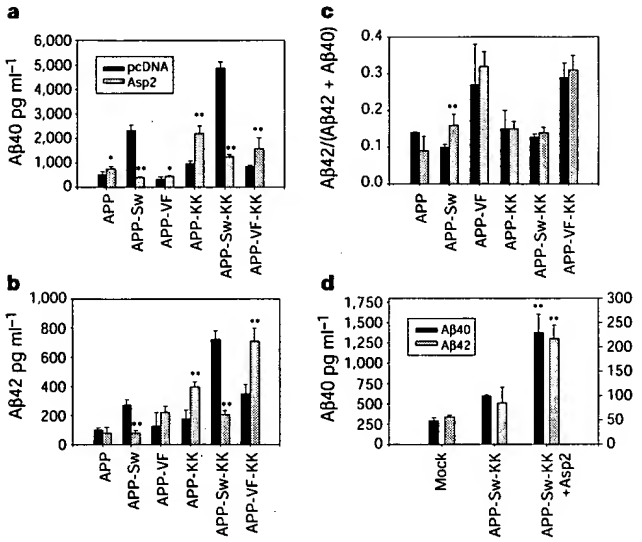

Figure 4 Asp2 increases Aβ peptide release. **a**, Production of Aβ40 from HEK293 cells transformed with Asp2 or empty vector DNA (pcDNA) subsequently transfected with the indicated APP constructs. **b**, Production of Aβ42 as in **a**. **c**, Ratio of Aβ42/(Aβ42+Aβ40) produced as in **a**. The APP constructs were transfected in quadruplicate cultures (asterisk, $P < 0.05$; double asterisk, $P < 0.001$). **d**, Production of Aβ40 and Aβ42 by IMR-32 cells co-transfected with APP-Sw-KK and either Asp2 or empty vector DNA.

cells transformed with the empty vector. This effect is cell-line-dependent as co-transfection of IMR-32 cells with vectors expressing Asp2 and APP-Sw-KK results in more than a twofold increase in the production of both Aβ40 and Aβ42 in comparison with cells co-transfected with the APP-Sw-KK construct and empty vector DNA (Fig. 4d). Differences in the level of Asp2 expression or its localizaton within HEK293 or IMR-32 cells may account for this difference.

We obtained direct evidence that Asp2 possesses β-secretase activity using biochemical studies that measured purified Asp2 proteolytic activity against synthetic APP peptide substrates. Native, full-length Asp2 was expressed in Chinese hamster ovary (CHO) cells. Its behaviour on cell fractionation, detergent solubilization and purification by sequential chromatography was consistent with that of an integral membrane protein. Sequence analysis of the purified recombinant protein indicated a major N-terminal sequence beginning with a glutamic acid (arrow in Fig. 1c); however, at present, it is unclear whether this is the N terminus of mature Asp2.

Two peptides were designed for assaying β-secretase activity. The first contained the wild-type APP β-secretase site, whereas the second contained the Swedish KM → NL modification of the β-secretase cleavage site. Maximal activity was seen with the Swedish β-secretase peptide. As expected for an aspartyl protease, proteolytic activity was sensitive to pH with maximal hydrolysis seen at pH 5.0. Amino-terminal sequencing of the two cleavage products verified that cleavage occurred at the sequence NL↓DA (Fig. 5a). The rate of cleavage was reduced tenfold in the corresponding wild-type APP peptide (Fig. 5b). Proteolytic activity was insensitive to 8 μM pepstatin or a mixture of 10 μM leupeptin, 10 μM E-64 and 5 mM EDTA, inhibitors of cathepsin D (and other aspartyl proteases), serine proteases, cysteinyl proteases, and metalloproteases, respectively. 'Mock' preparations of unmodified CHO cell membranes did not contain substantial Asp2-like activity. Thus, Asp2 acts directly in cell-free assays to cleave synthetic APP peptides at the β-secretase site, and the rate of cleavage is greatly increased by the Swedish KM → NL mutation associated with Alzheimer's disease.

Our experiments associated a new human aspartyl protease with the processing of APP at the β-secretase cleavage site. This protease

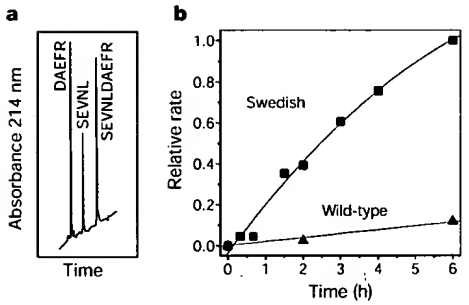

Figure 5 Asp2 β-secretase cleavage activity. **a**, Reverse phase HPLC profile showing the products of Asp2 cleavage. The amino-acid sequences of the parent peptide and the two hydrolysis products are indicated. **b**, Relative rates of hydrolysis of wild-type (triangles) and Swedish (squares) APP β-secretase peptides by Asp2.

contains an unusual C-terminal transmembrane domain that may help it colocalize with APP within cellular membranes where Aβ processing occurs. The expression pattern of Asp2 suggests a normal function in the brain, as well as in the exocrine pancreas. Our data indicate that Asp2 functions in the β-secretase pathway in cell lines of both somatic and neural origin, and that the enzyme meets many of the criteria expected of a APP β-secretase. Whether or not other enzymes also possess β-secretase activity is not excluded by these experiments. We note that identification of Asp2 as a candidate APP β-secretase has been reported independently by two other groups[17,18]. Thus, inhibitors of Asp2 will provide a new approach to the treatment of Alzheimer's disease. □

Methods

Reagents

Northern hybridization was performed using human tissue blots (Clontech). Chromosomal localization was performed by Genome Systems, Inc. *In situ* hybridization to human tissues was performed by LifeSpan Biosciences, Inc. We used 6E10 and 4G8 (Senetek), 22C11 (Boerhinger-Mannheim), LN27 (Zymed Laboratories) Rb162 and Rb165 (New York Institute for Basic Research), 369 (Paul Greengard), and C8 (Dennis Selkoe) antibodies. Oligofectin-G and the Asp2-1 (5′-CCCATAACAGTGCCCGTGGATGACT-3′) and Asp2-2 (5′-GAACTCATCGTGCACATGGCAAGCG-3′) chimeric antisense oligomers were from Sequitur, Inc. APP constructs were assembled in the vector pIRES (Clontech). Asp2 constructs were assembled in the vector pcDNA3.1/hybro (Invitrogen). The sequence for the HEX-labelled TaqMan probe (Perkin Elmer) was 5′-AGGGCAA CAACGACGCCGAATTACA-3′. Amplification was performed with the primer pair 5′-TCAGAGCAGCCAATGGCC-3′ and 5′-GCCTGTAGGTGGCTGGACA-3′.

Transfection and immunodetection

Oligofectin-G was used for lipid-mediated transfection of antisense oligomers into cultured cells according to the manufacturer's protocol. Supernatants and cell lysates were harvested 72 h after transfection. Transfection of plasmid DNA was performed using either the calcium phosphate method or Lipofectamine (GIBCO-BRL). The total amount of DNA used for transfection was held constant by adding empty vector DNA to the transfection mixture. Cell lysates and supernatants were harvested 48–160 h after transfection. Cells were lysed with 10 mM HEPES, pH 7.9, 150 mM NaCl, 10% glycerol, 1 mM EGTA, 1 mM EDTA, 0.1 mM sodium vanadate and 1% NP-40. Equal amounts of protein (50 μg) were resolved on 4–12% Tricine gels (Novex), and transferred to nitrocellulose membranes for probing with 6E10, 22C11 or C8 antibodies. For immunoprecipitation, equal amounts of protein corresponding to one plate of cells were incubated with C8 or 369 antibody at 4 °C overnight, captured with protein A/protein G agarose beads, and processed for western blot detection of CTF99 with 6E10. The Aβ EIA was done as described[19] using 6E10 monoclonal antibody as a capture antibody and biotinylated Rb162 or Rb165 antibody for detection of Aβ40 and Aβ42, respectively. The sAPPα EIA used LN27 antibody as a capture antibody and biotinylated 6E10 for detection.

Proteolytic activity assays

Recombinant Asp2 was purified from CHO cell membranes by solubilization in 25 mM Tris-HCl, pH 8.0, containing 50 mM β-octylglucoside followed by sequential chromatography on MonoQ and MonoS columns. Material prepared in this manner was more than 95% pure by SDS–PAGE analysis. Activity assays for Asp2 were performed using synthetic peptide substrates containing either the wild-type APP β-secretase site (SEVKM↓DAEFR) or the Swedish KM → NL mutation (SEVNL↓DAEFR). Reactions were performed in 50 mM 2-(*N*-morpholino)ethane-sulfonate, pH 5.5, containing 70 μM peptide substrate and recombinant Asp2 for various times at 37 °C. The reaction products were quantified by reverse phase HPLC. The sequence of both products was confirmed using Edman sequencing and MADLI-TOF mass spectrometry.